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Isolation and Identification Methods for *Slackia Exigua* and Investigation of the Relationship between this Organism and Peri-Implantitis

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Abstract

In the genus Slackia, *Slackia exigua* (formerly *Eubacterium exiguum*) is isolated from the human oral cavity. A relationship was recently reported between S. exigua and periodontal disease, including chronic periodontitis and peri-implantitis. A suitable selective medium for the isolation of *S. exigua* is needed in order to assess the veritable prevalence of this organism in various lesions of the oral cavity. The purpose of the present study was to develop selective media for the isolation of *S. exigua* and investigate whether the monitoring of *S. exigua* levels is useful as a clinical indicator for the diagnosis of peri-implantitis.

In order to examine the bacterium population in the oral cavity, a novel selective medium (SEXSM) was herein developed for the isolation of *S. exigua*. SEXSM consists of tryptic soy agar, yeast extract, hemin, Vitamin K1, L-cysteine, sheep blood, 2-phenylethanol, trimethoprim, nalidixic acid, and polymyxin B. The average growth recovery of S. exigua on SEXSM was 98.3% that on CDC blood agar. The growth of other representative oral bacteria, i.e., the genera Streptococcus, Actinomyces, Neisseria, Corynebacterium, Veillonella, Fusobacterium, and Rothia, was strongly inhibited on the selective medium. PCR primers were designed based on partial sequences of the 16S rDNA genes of *S. exigua*. These primers reacted with *S. exigua*, but not with other representative oral bacteria. These results indicate that these primers are useful for identifying *S. exigua*. The proportion of S. exigua in Gingival Crevicular Fluids (GCF) collected from periodontally Healthy with Implants (HI) and Peri-Implantitis (PI) groups was examined. Colonies on SEXSM were subcultured for confirmation by a PCR analysis using the primers designed in the present study. The growth recoveries of S. exigua strains on SEXSM were very satisfactory. *S. exigua* in GCFs of the HI and PI groups were detected at 0.002%, and 1.7% to the total bacteria number, respectively. The selective medium, designated SEXSM, and a PCR method using the primers designed in the present study were useful for the isolation and identification of *S. exigua*. Moreover, the monitoring of *S. exigua* levels was useful as a clinical indicator for the diagnosis of peri-implantitis.

Keywords

Slackia exigua; Selective medium; Oral cavity; Peri-implantitis

Introduction

The genus Slackia comprises 6 species (http://www.bacterio.net/slackia.html). The name "slackia" was adopted in honor of Geoffrey Slack, a distinguished British microbiologist and dental researcher. Wade, et al reported a 16S rRNA gene sequence similarity of 94.5% between Eubacterium exiguum from human oral lesions and Peptostreptococcus heliotrinreducens from the sheep rumen as well as a 60 to 64 mol% G+C content for the DNA base composition of the two species [1]. Based on these sequence similarity data, the new genus Slackia was created for these two bacterial species, and *E. exiguum* and *P. heliotrinreducens* were reclassified as Slackia exigua and S. heliotrinreducens, respectively. Phenotypic and phylogenetic analyses have since revealed four more species in the genus Slackia: *S. faecicanis*, *S. isoflavoniconvertens*, *S. equolifaciens*, and *S. piriformis*. S. exigua was isolated from human oral lesions; *S. heliotrinreducens* was isolated from the sheep rumen; *S. faecicanis* was isolated from dog feces; and *S. isoflavoniconvertens*, *S. equolifaciens*, and *S. piriformis* were isolated from human feces [2-7].

S. exigua has frequently been isolated from necrotic pulp and periradicular lesions [8-10]. These findings suggest that this species plays a pathogenic role in oral infectious

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diseases, including pulpal infections that spread to periradicular tissues. Furthermore, this organism has been isolated from other oral sites, human wound infections and abscesses, severe empyema with acute respiratory distress syndrome, polymicrobial feculent meningitis, and bacteremia [11-18]. A relationship has been reported between *S. exigua* and chronic periodontitis and peri-implantitis [19,20]. Thus, a suitable selective medium is needed in order to assess the veritable prevalence of *S. exigua*, which is capable of causing periodontal disease, including chronic periodontitis and peri-implantitis.

A sequence analysis of several target genes is the most reliable method for identifying bacterial species, but is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay, such as a Polymerase Chain Reaction (PCR) amplification method, for identifying *S. exigua* is needed. A 16S rDNA PCR amplification method is sensitive and useful [21].

The aims of the present study were to develop isolation and identification methods for *S. exigua* using selective medium and a PCR method, respectively, and to investigate whether the monitoring of *S. exigua* levels is useful as a clinical indicator for the diagnosis of peri-implantitis.

Materials and Methods

Bacterial Strains and Culture Conditions

All bacterial strains used in the present study are listed in Table 1. The anaerobic bacteria (i.e., S. exigua, Veillonella parvula, and Fusobacterium nucleatum) used in the present study were maintained by cultivating them on Anaerobic Blood Agar (CDC), which has a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K1 (10 μg/ml), hemin (5 μg/ml), L-cysteine (800 μg/ml), 0.5% yeast extract, and 5% sheep blood. These organisms were cultured at 37°C for 48 h in an anaerobic chamber (Forma Scientific Anaerobic System Model 1024, Forma Scientific, Marietta, OH, U.S.A) with 80% N2, 10% H2, and 10% CO2. S. exigua isolates (NUM-Sex 6965, NUM-Sex 6967, NUM-Sex 6969, NUM-Sex 6972, and NUM-Sex 6977) were obtained with non-selective medium, i.e., CDC, from the human oral cavity in our previous studies.

Strains other than anaerobic bacteria were maintained by cultivating them on BactTM Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). These organisms were cultured at 37°C overnight in an atmosphere of 5% CO2 in a CO2 incubator (NAPCO® Model 5400; Precision Scientific, Chicago, IL, USA).

Development of the New Selective Medium

Evaluation of the base medium: BHI supplemented with 1% yeast extract (BHI-Y), BHI-Y supplemented with 5% sheep blood (BHI-Y blood), and CDC were examined as the base medium in the selective medium. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates on which bacteria, except anaerobic bacteria, were inoculated were cultured at 37°C for 72 h in an atmosphere of 5% CO_2 in a CO_2 incubator, and the plates on which anaerobic bacteria were inoculated were cultured at 37°C for 6 days under anaerobic conditions. After cultivation, the number of colony-forming units (CFU)/ml was counted.

Susceptibility tests: Preliminary studies of antibiotic selection were also performed using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method was used for susceptibility testing [22].

Recovery of *S. exigua* and other Representative Oral Bacteria

The recoveries of the *S. exigua* reference strain, *S. exigua* isolates, and other representative oral bacteria were calculated as CFU/ml on selective medium and compared with those on CDC for total cultivable bacteria. All bacterial strains used in the present study are listed in Table 1.

All bacterial strains, except anaerobic bacteria, were pre-incubated in BHI broth at 37°C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator. Anaerobic bacteria were pre-incubated in Tryptic soy broth (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with vitamin K1 (10 μg/ml), hemin (5 μg/ml), and 0.5% yeast extract at 37°C overnight under anaerobic conditions. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates on which bacteria, except anaerobic bacteria, were inoculated were cultured at 37°C for 72 h in an atmosphere of 5% CO2 in a CO2 incubator, and the plates on which anaerobic bacteria were inoculated were cultured at 37°C for 5 days under anaerobic conditions. After cultivation, the number of CFU/ml was counted.

Clinical Samples

One hundred and twenty patients attending Nihon University Hospital, School of Dentistry at Matsudo, participated in the present study. They were divided into two subject groups: periodontally healthy with implants (HI) and Peri-Implantitis (PI) groups. Thirty HI and thirty PI subjects were selected by inclusion criteria for peri-implantitis as follows: patients who underwent dental implantation treatments between 2013 and 2016; patients with at least 1 dental implant for more than half a year; according to the Guidelines of Periodontology, peri-implantitis was defined as Bleeding of Probing (BOP) and/or periodontal pocket depth (PPD) ≥ 4 mm, accompanied by bone tissue loss under the first thread of the implant (i.e., bone absorption ≥ 2 mm). Exclusion criteria were as follows: patients with systematic diseases; patients receiving periodontal therapy within 6 months; taking immunosuppressive agents or antibiotics; the long-term use of contraceptive drugs; pregnant women.

Gingival Crevicular Fluid (GCF) was collected using endodontic paper points from all subjects and placed in a sterile microcentrifuge tube containing 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2). Samples were dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY., USA). Portions (100 μl) of appropriate dilutions of these samples were plated in triplicate on CDC and selective medium plates. CDC plates for total cultivable bacteria and selective medium plates were cultured at 37ºC for 5 days under anaerobic conditions. After cultivation, the number of CFU/ml was calculated. This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 17-014).

Identification of *S. exigua* Species Isolated from Clinical Samples

Twenty-four of the approximately 50 colonies that grew on the selective medium plate per subject were randomly isolated, subcultured, and their identity was then confirmed by a PCR analysis.

Design of Species-Specific Primers for *S. exigua*

The design of species-specific primers for S. exigua was performed as follows. Briefly, the 16S rRNA sequences of *S. exigua* (accession no. AF101240), *S. heliotrinreducens* (accession no. CP001684), *S. faecicanis* (accession no. AJ608686), *S. isoflavoniconvertens* (accession no. EU826403), *S. equolifaciens* (accession no. EU377663), and *S. piriformis* (accession no. AB490806) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and multiple sequence alignment analyses were performed using the CLUSTAL W program; i.e., the 16S rRNA sequences of six Slackia species were aligned and analyzed. Homologies among the primers selected for S. exigua were confirmed by a BLAST search.

Development of a PCR Method for Identifying *S. exigua* using Designed Primers

A PCR method for identifying S. exigua using the designed primers was developed as follows. Bacterial cells were cultured in BHI broth overnight, and 1 ml of the sample was then collected in a microcentrifuge tube and resuspended at a density of 1.0 McFarland standard (approximately 107 CFU in 1 ml of sterile distilled water). A total of 3.6 μl of the suspension was then used as a PCR template.

Strain	CDC blood	SEXSM	Recovery %
	CFU/ml × 10^8	CFU/ml × 10^8	
Slackia exigua			
JCM 11022	1.5 ± 0.2[a]	1.5 ± 0.3	99.3
NUM-Sex 6965	2.2 ± 0.3	2.1 ± 0.2	98.9
NUM-Sex 6967	1.0 ± 0.3	1.0 ± 0.3	99.1
NUM-Sex 6969	1.1 ± 0.2	1.1 ± 0.3	98.0
NUM-Sex 6972	0.7 ± 0.3	0.7 ± 0.2	96.6
NUM-Sex 6977	0.9 ± 0.2	0.9 ± 0.3	98.1
Streptococcus oralis			
ATCC 10557	1.5	0	0
Streptococcus salivarius			
JCM 5707	2.8	0	0
Streptococcus anginosus			
ATCC 33397	3.1	0	0
Streptococcus mutans			
ATCC 25175	5.5	0	0
Streptococcus sobrinus			
ATCC 33478	7.4	0	0
Actinomyces naeslundii			
ATCC 12104	1.2	0	0
Actinomyces oris			
ATCC 27044	2.5	0	0
Actinomyces odontolyticus			
ATCC 17929	2.2	0	0
Corynebacterium matruchotii			
ATCC 14266	1.9	0	0
Corynebacterium durum			
ATCC 33449	0.9	0	0
Rothia dentocariosa			
JCM 3067	0.8	0	0
Rothia mucilaginosa			
JCM 10910	0.7	0	0
Rothia aeria			
JCM 11412	1.1	0	0
Veillonella parvula			
ATCC 10790	1.1	0	0
Fusobacterium nucleatum			
ATCC 22586	2.1	0	0
N. sicca			
ATCC 29256	0.7	0	0

Table 1: Recovery of *S. exigua* and other bacteria on CDC blood and SEXSM

[a]Ave ± SD.

The detection limit of PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 2 µM of each primer, 10 µl of 2 × MightyAmp Buffer Ver.2 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 3.6 µl of the template in a final volume of 20 µl. PCR was performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Carlsbad, CA). PCR conditions included an initial denaturation step at 98°C for 2 min, followed by 30 cycles consisting of 98°C for 10 s and 68°C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1 × Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker.

Statistical Analysis

The proportions of S. exigua to the total bacteria number in GCFs from the HI and PI groups were compared by the Kruskall-Wallis test (Mann-Whitney U test). Values of $P < 0.05$ were considered to be significant.

Results

Development of the Selective Medium

Selection of the base medium: The selection of a base medium for the growth of S. exigua was performed. *S. exigua* grew well on CDC, but did not grow well and showed extremely small colonies on BHI-Y and BHI-Y blood (data not shown). CDC was ultimately selected as the base medium.

Susceptibility to antibiotics: *S. exigua* was more resistant to 2-phenylethanol than oral Gram-negative rods, such as Fusobacterium species. The minimal inhibitory concentration (MIC) of 2-phenylethanol for *S. exigua* was 10 µl/ml. Oral Gram-negative bacteria were sensitive to 1.5 µl/ml of 2-phenylethanol. *S. exigua* was more resistant to nalidixic acid than oral Gram-negative *cocci*, such as *Neisseria* and *Veillonella* species. The minimal inhibitory

concentration (MIC) of nalidixic acid for *S. exigua* was 200 µg/ml. Oral Gram-negative bacteria were sensitive to 30 µg/ml of nalidixic acid. *S. exigua* was more resistant to trimethoprim than oral streptococcus species, oral Corynebacterium species, and oral Actinomyces species (*Actinomyces naeslundii, A. oris,* and *A. odontolyticus*) other than *A. israelii* and *A. meyeri*. The MIC of trimethoprim for *S. exigua* was 5000 µg/ml. Oral streptococcus species, *oral Corynebacterium species*, and oral Actinomyces species other than *A. israelii* and *A. meyeri* were sensitive to 125 µg/ml of trimethoprim. *S. exigua* were more resistant to polymyxin B than A. israelii and A. meyeri. The MIC of polymyxin B for *S. exigua* was 100 µg/ml. *A. israelii* and *A. meyeri* were sensitive to 5 µg/ml of polymyxin B.

Composition of the new selective medium: The new selective medium, designated *S. exigua* selective medium (SEXSM), was composed of the following (per liter): 40 g of tryptic soy agar, 5 g of yeast extract, 5 mg of hemin, 10 mg of Vitamin K1, 800 mg of L-cysteine, 50 ml of sheep blood, 1.5 ml of 2-phenylethanol, 1000 mg of trimethoprim, 30 mg of nalidixic acid, and 5 mg of polymyxin B. Sheep blood, 2-phenyletthanol, and antibiotics, i.e., trimethoprim, nalidixic acid, and polymyxin B, were added after the base medium had been sterilized and cooled to 50°C.

PCR Method for Identifying *S. exigua*

Primer design: The specific primer set covering the upstream region of the 16S rDNA sequence of *S. exigua* was designed in the present study (Table 2). The amplicon size of *S. exigua* was 739 bp.

Detection limit: A PCR method was used to identify the *S. exigua* -amplified DNA fragment of the expected size for this organism (Figure 1). The detection limit was assessed in the presence of titrated bacterial cells, and the detection sensitivity of the PCR assay was 50-100 CFU per PCR template (5.6 µl) for the S. exigua -specific primer set with strain JCM 11022 (data not shown).

Assay of *S. exigua* and Representative Oral Acteria: The PCR method used to identify *S. exigua* produced positive bands from the S. exigua reference strain JCM 11022 and *S. exigua* clinical isolate NUM-Sex 6965, and did not produce any amplicons from other Slackia species (Figure 1).

Some *Streptococci, Actinomyces, Neisseria, Corynebacterium, Rothia, Veillonella*, and *Fusobacterium* species were subjected as representative oral bacteria to PCR using the designed primer set. No amplicons were produced from any of the representative oral bacteria (Figure 1).

Recovery of *S. exigua* and Inhibition of Other Representative Oral Bacteria on Selective Medium

Table 1 shows the recovery of the *S. exigua* reference strain JCM 11022 and isolates on SEXSM relative to CDC. The growth recoveries of the *S. exigua* reference strain and isolates ranged between 96.6% and 99.3% (average 98.3%) on SEXSM relative to that on CDC.

Table 1 also shows the inhibition of other representative oral bacteria on SEXSM relative to CDC agar. The growth of other representative oral bacteria was markedly inhibited on selective medium. Furthermore, the growth of *S. faecicanis* JCM 14555, *S. equolifaciens* JCM 16059, *S. piriformis* JCM 16070, and *S. isoflavoniconvertens* JCM 16137 was inhibited on SEXSM (data not shown).

Clinical Examination

The detection frequencies of *S. exigua* in GCFs from the 4 groups are shown in Table 3. In GCFs from the HI (n=30) and PI (n=30) groups, S. exigua was detected at 33.3%, and 100%, respectively. The mean numbers of total cultivable bacteria and *S. exigua* in the HI group were 1.0×104 CFU/ml (range: 2.2 × 103 - 3.6 × 104) and 1.8 × 10-1 CFU/ml (range: 0 - 1.8 × 10), respectively; and those in the PI group were 2.0 × 105 CFU/ml (range: 2.0 × 104 - 1.2 × 106) and 3.4 × 103 CFU/ml (range: 3.4 × 102 - 2.0 × 104), respectively. Comparisons of *S. exigua* proportions to the total bacteria number in GCFs between the 2 groups are shown in Figure 2. *S. exigua* in GCFs from the HI and PI groups accounted for 0.002% and 1.7% of all bacteria, respectively. The proportion of S. exigua to the total bacteria number in GCF was significantly higher in the PI group than in the HI group *(P < 0.001)*.

In the first isolation, *S. exigua* colonies on SEXSM commonly had a smooth and circular appearance. The colony color and average colony size of *S. exigua* on SEXSM was light purple and 1.0 mm in diameter, respectively (Figure 3).

Species	Primer	Sequence	Product size (bp)	Position	Accession number
S. exigua	SEXF	TGCCTGCTGCATGGTGGGTG	740	55-73	AF101240
	SEXR	AAAGGGACAGGCCTGCTTC		794-775	

Table 2: Locations and sequences of species-specific primers for the16S rDNA of *S. exigua*

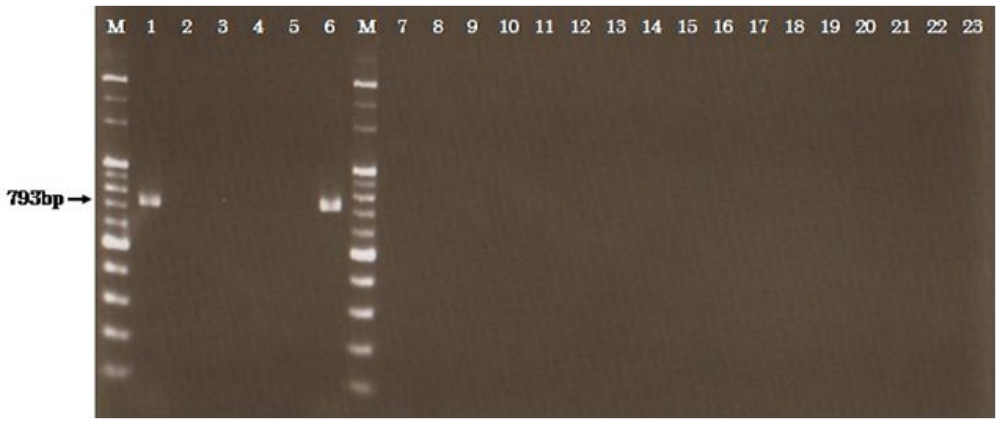

Figure 1: Primers are a mixture of SEXF and SEXR.

Lanes: 1, *S. exigua* JCM 11022; 2, *S. faecicanis* JCM 14555; 3, *S. equolifaciens* JCM 16059; 4, *S. piriformis* JCM 16070; 5, *S. isoflavoniconvertens* JCM 16137; 6, *S. exigua* clinical isolate NUM-Sex 6965; 7, *Streptococcus mitis* ATCC 49456; 8, *S. oralis* ATCC 10557; 9, *S. sanguinis* ATCC 10556; 10, *S. salivarius* JCM 5707; 11, *S. anginosus* ATCC 33397; 12, *S. sobrinus* ATCC 33478; 13, *S. mutans* NCTC 10449; 14, *A. naeslundii* ATCC 12104; 15, *A. oris* ATCC 27044; 16, *A. odontolyticus* ATCC17929; 17, *R. dentocariosa* JCM 3067; 18, *R. mucilaginosa* JCM 10910; 19, *Corynebacterium matruchotii* ATCC 14266; 20, *C. durum* ATCC 33449; 21, *Neisseria sicca* ATCC 29256; 22, *V. parvula* JCM 10790; 23, *F. nucleatum* ATCC 22586. M, molecular size marker (100-bp DNA ladder).

Group	Subject		No. of *S. exigua* positive samples (%, frequency)	No. of total bacteria	Total no. of *S. exigua*
	No. (male: female)	Average age (range)		CFU/ml	CFU/ml
Periodontally healthy with implants (n=30)	30 (12:18)	51 (40-69)	10 (33.3)	1.0×10^{4}	1.8×10^{-1}
Peri-implantitis (n=30)	30 (14:16)	63 (51-85)	30 (100.0)	2.0×10^{5}	3.4×10^{3}

Table 3: Frequency of *S. exigua* in GCFs from 2 groups

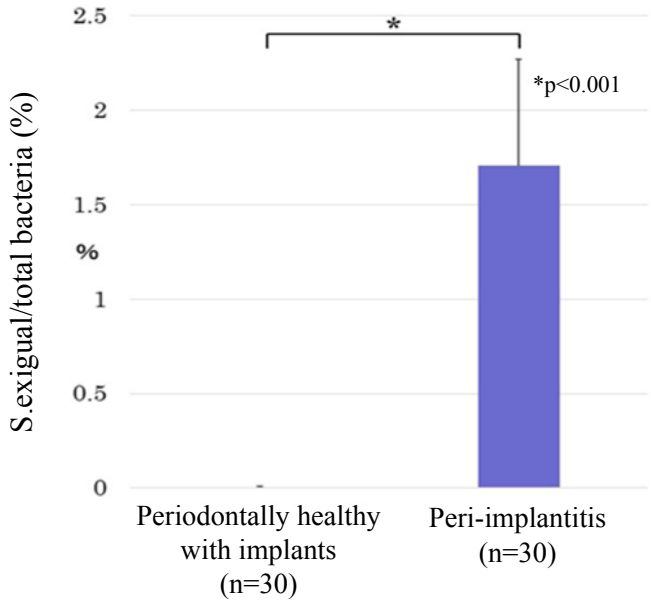

Figure 2: Comparison of *S. exigua* proportions in GCFs from 2 groups.

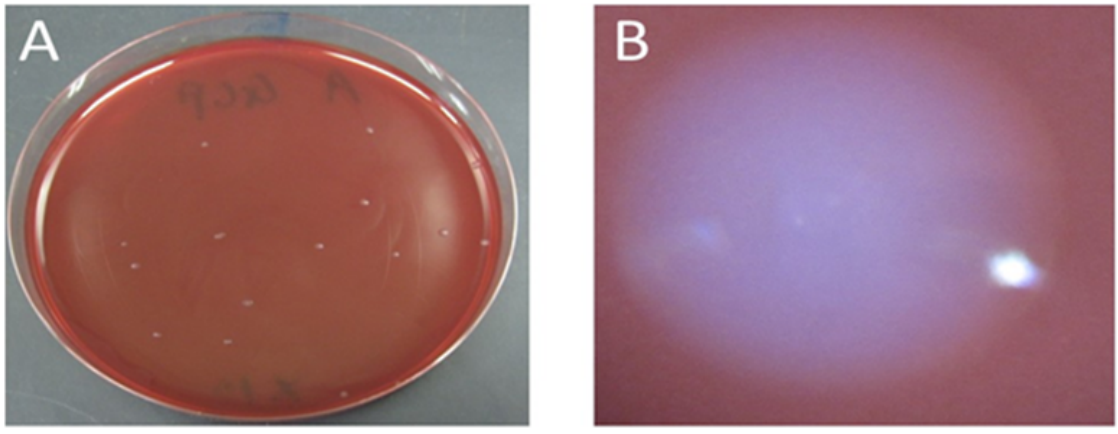

Figure 3: Appearance of *S. exigua* colonies on SEXSM. A) *S. exigua* colonies on SEXSM inoculated with a GCF sample. B) Stereomicroscope image of *S. exigua* colonies on SEXSM.

Discussion

S. exigua is a non-sporing, non-motile, strictly anaerobic, asaccharolytic, and Gram-positive rod. Amino acids are important metabolic substrates for the growth of this organism, particularly arginine and lysine, which are produced by the enzymatic degradation of peptides by trypsin-like proteinases. *S. exigua* does not produce butyrate from ornithine, which may be one of the reasons for its poor growth [23].

Asaccharolytic and Gram-positive rods, including *S. exigua*, use peptides for their growth, and may be associated with proteolytic activities in sites of inflammation that are diseased. *S. exigua* was isolated from various oral sites, such as infected dental pulps, infected periradicular tissues, periodontal pockets with chronic periodontitis, and acute dento-alveolar abscesses [8-10,12,13,24,25]. A relationship has been reported between *S. exigua* and peri-implantitis [20]. Dental implant-based reconstruction, which has been adapted to replace conventional fixed or removable partial dentures, has led to the emergence of peri-implantitis as a serious problem in 28–56% of recipients and a major cause of implant loss [26]. As such, effective prevention and management of peri-implantitis are essential for

improving the quality of life and health of patients. Thus, a suitable selective medium and reliable identification method are needed in order to assess the veritable prevalence of *S. exigua*, which is capable of causing peri-implantitis.

In the present study, we designed species-specific primers to identify *S. exigua* using a PCR method. These primers were able to distinguish *S. exigua*, and did not react with representative oral bacteria or other Slackia species. Moreover, the PCR method in this study directly uses bacterial cells with Mighty Amp DNA Polymerase Ver.2 (Takara) and is completed within approximately 2 hours.

A useful selective medium for isolating *S. exigua* may contribute to the correct and rapid diagnosis of infectious diseases caused by this organism. However, a selective medium that is useful for the isolation of *S. exigua* did not exist. In the present study, the *S. exigua* strains used were more resistant to 2-phenyletthanol, trimethoprim, nalidixic acid, and polymyxin B than other oral bacteria.

The growth of oral bacteria detected in the oral cavity was inhibited by the addition of 1.5 ml/L 2-phenylethanol, 1000 mg/L trimethoprim, 30 mg/L nalidixic acid, and 5 mg/L polymyxin B to CDC agar. All of the S. exigua reference strains and isolates tested grew well on the new selective medium, designated as SEXSM, while the growth of other bacteria was markedly inhibited (Table 1). Moreover, SEXSM easily distinguished *S. exigua* by its characteristic colony morphology. However, other than *S. exigua, S. faecicanis, S. equolifaciens, S. piriformis* and *S. isoflavoniconvertens* did not grow on SEXSM (data not shown).

The distribution of *S. exigua* in the oral cavity of humans has not yet been reported in detail. In the present study, *S. exigua* in GCFs from the HI and PI groups was detected at 33.3% and 100%, respectively. Furthermore, the proportion of S. exigua to the total bacteria number in GCF was significantly higher in the PI group than in the HI group ($P < 0.001$). These results indicate that the monitoring of *S. exigua* levels is useful as a clinical indicator for the diagnosis of peri-implantitis. We developed a selective medium, designated SEXSM, to isolate S. exigua in the oral cavity of humans. Since SEXSM is highly selective for *S. exigua*, it will be useful for assessing the distribution and role of this organism at various locations in humans.

Selective medium (SEXSM) and a PCR method as isolation and identification methods for S. exigua may contribute to the diagnosis of infectious diseases, including peri-implantitis, which are caused by this organism.

Author's Contributions

TK, HG, TT, HT and KU corrected the data. TK, SU, OT MO, and CU drafted and wrote the manuscript. The concept of this manuscript was devised by TK. All authors read and approved the final manuscript.

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